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SOX9 Enhances Aggrecan Gene Promoter/Enhancer Activity and Is Up-regulated by Retinoic Acid in a Cartilage-derived Cell Line, TC6*

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SOX9 is a transcription factor that plays a key role in chondrogenesis. Aggrecan is one of the major structural components in cartilage; however, the molecular mechanism of aggrecan gene regulation has not yet been fully elucidated. TC6 is a clonal chondrocytic cell line derived from articular cartilage. The purpose of this study was to examine whether SOX9 modulates aggrecan gene expression and to further identify molecules that regulate *Sox9* expression in TC6 cells. SOX9 overexpression in TC6 cells enhanced by ~3-fold the transcriptional activity of the AgCAT-8 construct containing 8-kilobase (kb) promoter/first exon/first intron fragments of the aggrecan gene. SOX9 enhancement of aggrecan promoter activity was lost when we deleted a 4.5-kb fragment from the 3′-end of the 8-kb fragment corresponding to the region including the first intron. In TC6 cells, SOX9 enhanced the transcriptional activity of a reporter construct containing the *Sry/Sox* consensus sequence >10-fold. SOX9 enhancement of aggrecan gene promoter activity and SOX9 transactivation through the *Sry/Sox* consensus sequence were not observed in osteoblastic osteosarcoma cells (ROS17/2.8), indicating the dependence on the cellular background. Northern blot analysis indicated that TC6 cells constitutively express *Sox9* mRNA at relatively low levels. To examine regulation of *Sox9* gene expression, we investigated the effects of calciotropic hormones and cytokines. Among these, retinoic acid (RA) specifically enhanced *Sox9* mRNA expression in TC6 cells. The basal levels of *Sox9* expression and its enhancement by RA were observed similarly at both permissive (33 °C) and nonpermissive (39 °C) temperatures. Furthermore, RA treatment enhanced the transcriptional activity of a reporter construct containing the *Sry/Sox* consensus sequence in TC6 cells. Moreover, RA treatment also enhanced the transcriptional activity of another reporter construct containing the enhancer region of the type II procollagen gene in TC6 cells. These observations indicate that SOX9 enhances aggrecan promoter activity and that its expression is up-regulated by RA in TC6 cells.

Sox9 is a member of the family of *Sox* (*Sry*-type high mobility group box) genes that were first identified on the basis of a region with high homology to that of *Sry* (sex-determining region Y) (1). This region encodes a 79-amino acid motif that is known as a high mobility group box and is responsible for sequence-specific binding to DNA (2, 3). Several high mobility group box proteins are known to act as transcription factors (4), and some of the *Sox* genes have been shown to be expressed in a tissue-specific manner during development. SOX9 is expressed predominantly in cells in mesenchymal condensations during the early development of skeletons in embryos (5). These SOX9-expressing regions coincide with those where deposition of cartilage matrix takes place, suggesting a role for SOX9 in skeletal formation (6–8). In addition, mutations in human *SOX9* have been observed in patients with campomelic dysplasia that is characterized by the presence of skeletal malformation and XY sex reversal (9, 10). In mouse chimeras, $SOX9^{-/-}$ cells are excluded from all cartilaginous tissues during embryonic development and are present in juxtaposed mesenchyme that does not express chondrocyte-specific markers such as procollagen types II, IX, and XI and aggrecan (11). These observations indicate that SOX9 plays a key role in chondrogenesis and skeletogenesis.

Aggrecan, a large chondroitin sulfate proteoglycan, is one of the major structural components in cartilage matrix (12). Mice with a phenotype known as cartilage matrix deficiency (Cmd) have been found to possess an autosomal recessive mutation in the aggrecan gene. These mice exhibit cleft palate and short limbs, tail, and snout, whereas their levels of type II collagen and link protein are normal (13). The presence of the mutation in the aggrecan gene in the Cmd mice confirms the critical role of aggrecan in cartilage formation; however, the molecular mechanism of aggrecan gene regulation has not yet been fully elucidated.

TC6 cells are derived from articular cartilage of transgenic mice harboring a temperature-sensitive simian virus 40 large T-antigen gene (14). TC6 cells express mRNAs of the genes encoding cartilage phenotype-related proteins such as type II procollagen, link protein, and aggrecan.

The purpose of this study was to examine the effects of SOX9 on the aggrecan gene promoter/enhancer and regulation of *Sox9* gene expression in TC6 cells. We found that SOX9 enhances the promoter/enhancer activity of the aggrecan gene in TC6 cells. Furthermore, we also found that SOX9 expression is enhanced by RA[1] in TC6 cells.

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[1] The abbreviations used are: RA, retinoic acid; α-MEM, α-modified Eagle's medium; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); LUC, luciferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

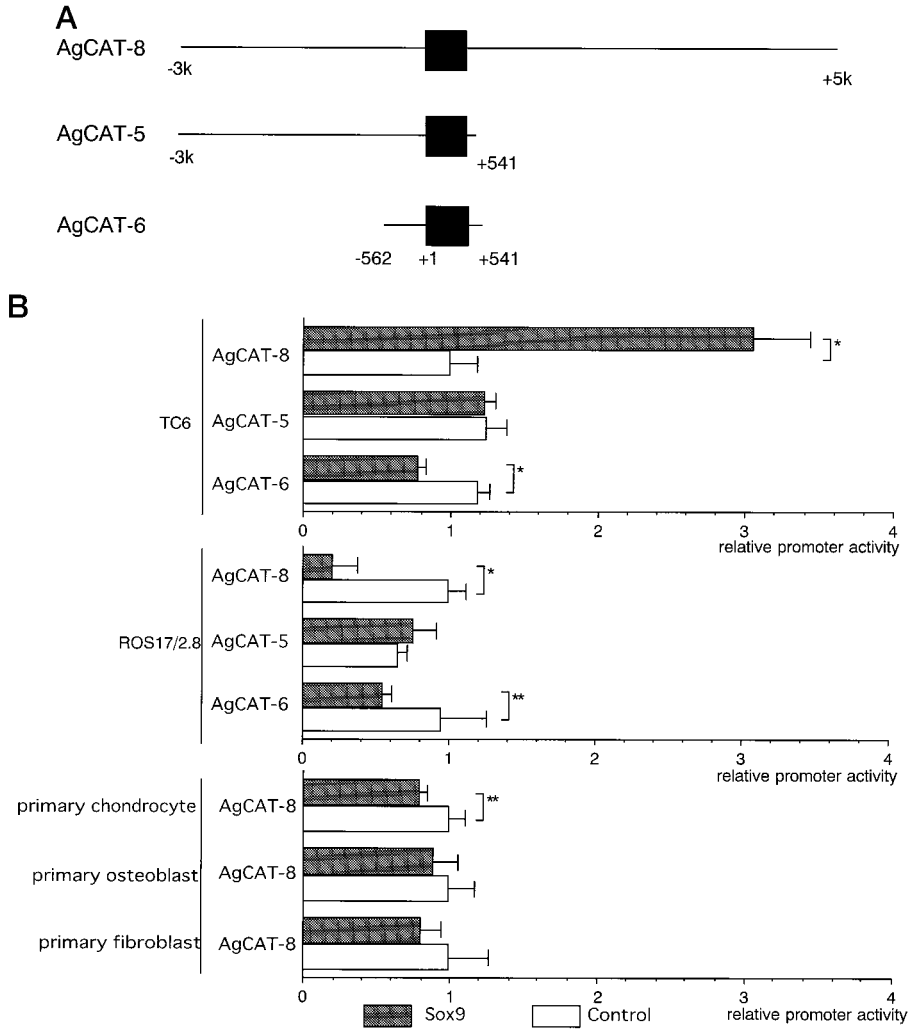

FIG. 1. **SOX9 enhances the transcriptional activity of the aggrecan gene via the enhancer region within the first intron in TC6 cells.** *A*, schematic illustration of the constructs of the aggrecan gene fragments linked to the CAT reporter gene. The AgCAT-8, AgCAT-5, and AgCAT-6 constructs contain 8-, 3.5-, and 1.1-kb aggrecan gene fragments, respectively. *Black boxes* indicate exons. *B*, SOX9 activation of the aggrecan-CAT reporter gene constructs in TC6 cells (*upper panel*); ROS17/2.8 cells (*middle panel*); and primary chondrocytes, primary osteoblasts, and primary fibroblasts (*lower panel*). These cells were cotransfected with 1 μg of reporter constructs and 1 μg of SOX9 expression vector or 1 μg of empty pSG5 vector. Transfected cells were incubated for 72 h, and CAT activity was determined as described under "Materials and Methods." Experiments were repeated three times (*upper* and *middle panels*) or twice (*bottom panel*) in triplicate with independent preparations of cell extracts. The representative data are shown. Data are presented as means ± S.D. ($n = 3$). *, $p < 0.01$; **, $p < 0.05$ by unpaired Student's t test.

MATERIALS AND METHODS

Cell Culture—TC6 cells were cultured at 33 °C in α-modified Eagle's medium (α-MEM; Sigma) supplemented with 0.5% fetal bovine serum (FBS; Life Technologies, Inc.) (14). For temperature shift experiments, TC6 cells were cultured at 39 °C. ROS17/2.8 cells were cultured at 37 °C in modified Ham's F-12 medium (Life Technologies, Inc.) supplemented with 5% FBS (15). Primary chondrocytes were prepared from rib cartilage of newborn ICR mice (16). Chondrocytes were isolated from rib cage cartilage by digestion in 3 mg/ml collagenase (Sigma) for 30 min and then by further digestion in 3 mg/ml collagenase for 5 h. Primary osteoblasts were isolated from newborn ICR mouse calvariae by five sequential enzymatic digestions using 1 mg/ml collagenase and 250 units/ml Dispase (Godo Shusei, Tokyo, Japan) (17). The cells in the last three fractions were pooled. Primary fibroblasts were prepared from adult ICR mouse peritoneum. Peritoneum was incubated in 1 mg/ml collagenase and 250 units/ml Dispase for 1 h. Primary chondrocytes were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), and primary osteoblasts and primary fibroblasts were cultured in α-MEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) at 37 °C.

Chloramphenicol Acetyltransferase (CAT) Assay—CAT assay was performed as described previously (18). TC6 cells were plated in six-well cluster plates (35-mm well diameter) at 6–7 × 10^4 cells/cm^2. One day later, TC6 cells were cotransfected with 1 μg of CAT reporter plasmids and a SOX9 expression plasmid containing a 1.9-kb *Sox9* cDNA insert (5) or a pSG5 vector plasmid as a negative control. The reporter constructs AgCAT-8, AgCAT-5, and AgCAT-6, containing 8-, 3.5-, and 1.1-kb promoter/first exon (encoding the 5′-untranslated region)/first intron fragments of the mouse aggrecan gene, respectively (19), were used. TC6 cells were also transfected with 1 μg of pCII4-C reporter plasmid containing a 1.6-kb intron fragment of the murine type II procollagen gene (20). In some experiments, the cells were cultured in the presence or absence of 1 μM RA after transfection. Transfection was performed using LipofectAMINE (Life Technologies, Inc.) to form DNA-lipid complexes (21). TC6 cells were exposed to the DNA solution for 3 h without FBS, and then the cells were cultured in α-MEM supplemented with 0.5% FBS. ROS17/2.8 cells were subjected to transfection with the SOX9 expression vector in combination with reporter plasmids as described above. The cells were harvested 72 h after transfection. Cell extracts were prepared and used for the analysis of CAT activity. Protein concentrations in the cell lysates were determined according to the Coomassie Brilliant Blue G method as described (22). Equivalent amounts of the cellular proteins were incubated for 2 h at 37 °C in reaction buffer containing 0.25 M Tris-HCl (pH 7.5), 40 mM acetyl-CoA (Sigma), and [^{14}C]chloramphenicol (Amersham Pharmacia Biotech). The levels of acetylation were estimated by TLC followed by autoradiography of the TLC plates. Quantitation of the acetylation levels was performed using a BAS 2000 bioimaging analyzer system (Fuji Film Inc., Tokyo). The effect of *Sox9* promoter activity was estimated by measuring the conversion rate. Experiments were repeated two or three times in triplicate with independent preparations of cell extracts. To monitor transfection efficiency, CAT activity was normalized against the luciferase (LUC) activity of the pGL2-Control-LUC construct cotransfected with the reporter plasmids and SOX9 expression plasmid in part of the experiments. Such experiments gave results similar to those in which CAT activity was normalized against total protein contents.

Luciferase Assay—Transfection experiments were conducted as described above. The SOX9 effect on promoter activity was examined by measuring luciferase activity using a control empty vector plasmid (pSG5) as a reference. Luciferase assay was performed as described previously (23) using a Picagene kit (Tokyo Ink Co., Tokyo) and a luminometer (Berthold Autolumat LB953). TC6 cells were cotransfected with 1 μg of LUC reporter construct that carries four tandem copies of the 30-mer (GATCAGACTGAGAACAAAGCGCTCTCACAC) derived from the enhancer region of CD3-ϵ (24) containing the *Sry*/*Sox*

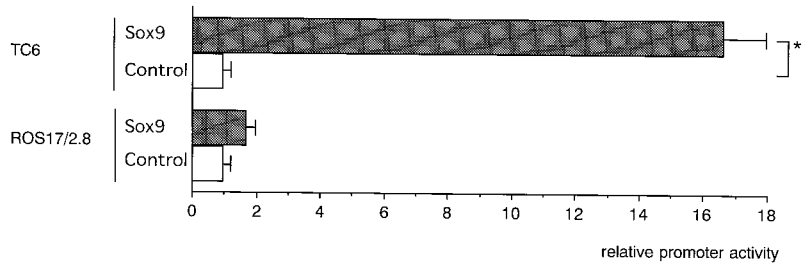

FIG. 2. **SOX9 transactivates via the *Sry/Sox* consensus sequence in TC6 cells, but not in ROS17/2.8 cells.** SOX9 modulation of the transcriptional activity of the AACAAAG concatamer-LUC reporter gene construct was examined. TC6 and ROS17/2.8 cells were cotransfected with 1 μg of reporter plasmid and 1 μg of SOX9 expression plasmid or 1 μg of empty pSG5 vector. The reporter plasmid carries four copies of the 30-mers containing AACAAAG. Experiments were repeated twice using six independently transfected cultures each time. Representative data are shown. Data are presented as means ± S.D. ($n = 6$). *, $p < 0.01$ by unpaired Student's t test.

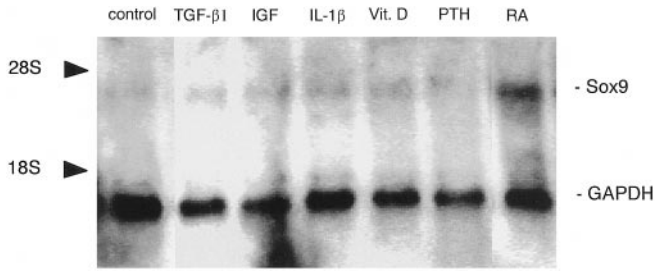

FIG. 3. ***Sox9* mRNA is constitutively expressed and is up-regulated by RA in TC6 cells.** Confluent TC6 cells were cultured for 48 h in the absence (*control*) or presence of 4 ng/ml transforming growth factor β1 (*TGF-β1*), 10 ng/ml insulin-like growth factor (*IGF*), 10 ng/ml interleukin-1β (*IL-1β*), 100 nM vitamin D (*Vit. D*), 100 nM parathyroid hormone (*PTH*), and 1 μM RA. Total RNA was isolated and subjected to Northern blot analysis as described under "Materials and Methods." The positions of *Sox9*, GAPDH, and 18 S and 28 S ribosomal RNAs are indicated. Experiments were repeated twice, and representative data are shown.

consensus sequence (AACAAAG) and the SOX9 expression vector or pSG5 vector plasmid. LUC activity was normalized against the total protein concentration measured by the Coomassie Brilliant Blue G method (22). In part of the experiments, the pSV2-CAT construct was cotransfected with LUC constructs to monitor transfection efficiency. These experiments gave results similar to those in which LUC activity was normalized against total protein contents. pGL2-Control-LUC (Promega) was used as a control.

Northern Blot Analysis—Total cellular RNA was prepared according to the acid/guanidium thiocyanate/phenol/chloroform method (25). Aliquots of 10 μg of the total RNA/lane were electrophoresed on 1.0% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters (Hybond-N, Amersham Pharmacia Biotech) by electroblotting. Filters were prehybridized for 24 h at 42 °C. A 1.6-kb *Eco*RI-*Bgl*II fragment of the mouse *Sox9* cDNA was used as a probe (5). A rat liver/bone/kidney-type alkaline phosphatase probe (0.6 kb) was prepared by digestion with *Eco*RI (26). A 1.2-kb *Eco*RI fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as a control. Each cDNA was labeled using the BcaBEST random primer labeling kit (Takara Shuzo Co., Ltd., Tokyo) and [α-^{32}P]dCTP (NEN Life Science Products). Hybridization was performed at 42 °C for 24 h. Filters were washed in 2× SSC and 0.5% SDS for 20 min at room temperature and in 0.2× SSC and 0.5% SDS for 20 min at 65 °C. Filters were then exposed to x-ray films using intensifying screens at −80 °C.

Growth Rate Evaluation—TC6 cells were seeded into four-well plates (2 cm^2/well) and cultured in α-MEM supplemented with 0.5% FBS in the absence or presence of RA. At the indicated time points, the cells were trypsinized and counted using a Coulter counter.

Statistical Analysis—Statistical evaluations of the data were conducted using unpaired Student's t test. Data are presented as means ± S.D. p values <0.05 were considered to be statistically significant.

RESULTS

SOX9 Enhances the Transcriptional Activity of the Aggrecan Gene Promoter/Enhancer in TC6 Cells—To examine whether SOX9 regulates the transcriptional activity of the aggrecan gene, a CAT reporter gene construct (AgCAT-8) containing an

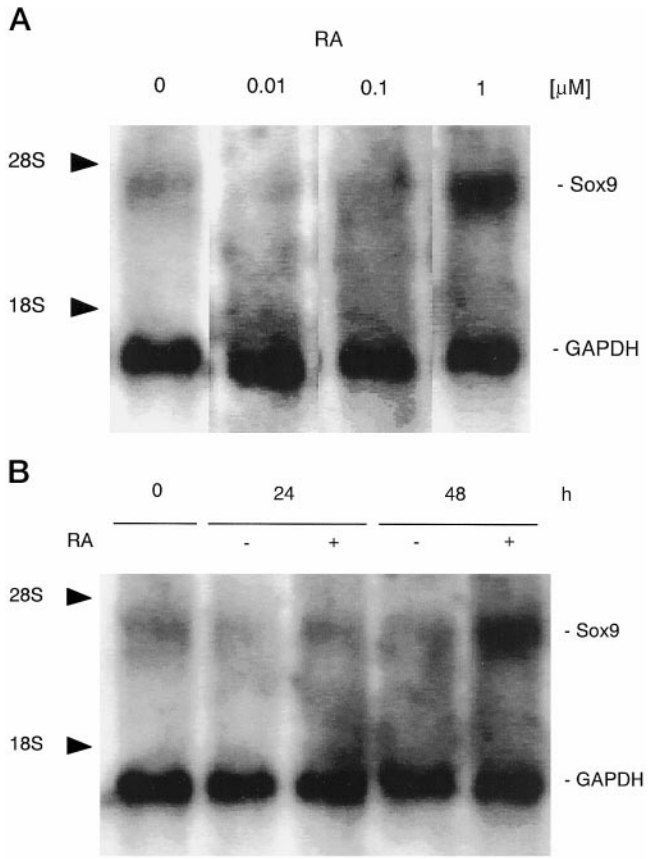

FIG. 4. **Dose and time effect of RA on *Sox9* mRNA expression in TC6 cells.** *A*, dose dependence of RA effect on the expression of *Sox9* mRNA. Confluent TC6 cells were treated with the indicated doses of RA for 48 h. *B*, time course of RA effect on the expression of *Sox9* mRNA. Confluent TC6 cells were cultured for the indicated hours in the absence (−) or presence (+) of 1 μM RA. Total RNA was prepared and subjected to Northern blot analysis as described under "Materials and Methods." The positions of *Sox9*, GAPDH, and 18 S and 28 S ribosomal RNAs are indicated. Experiments were repeated twice, and representative data are shown.

8-kb mouse aggrecan gene fragment that includes the aggrecan gene promoter, the first exon, and part of the first intron (Fig. 1*A*) was cotransfected (19) with a SOX9 expression vector into TC6 cells. SOX9 overexpression enhanced the transcriptional activity of the AgCAT-8 construct ~3-fold (Fig. 1*B*, *upper panel*). To further determine whether any particular region within the 8-kb aggrecan gene fragment responds to SOX9 overexpression, we also examined the CAT activities of the two deletion constructs AgCAT-5 (3.5 kb) and AgCAT-6 (1.1 kb) (Fig. 1*A*). SOX9 enhancement was no longer observed when we deleted a 4.5-kb intron fragment from the 3′-end of the 8-kb fragment corresponding to the region including the first intron

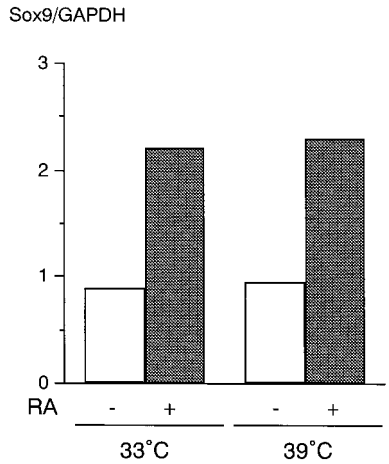

FIG. 5. **Temperature shift effect on *Sox9* mRNA expression in response to RA in TC6 cells.** TC6 cells were plated at 10^5/cm^2 and cultured at 33 °C for 24 h. Subsequently, TC6 cells were cultured for 48 h in the absence or presence of 1 μM RA at 33 or 39 °C. Total RNA was prepared and subjected to Northern blot analysis as described under "Materials and Methods." Quantitation was carried out using a densitometer, and the ratios of *Sox9* to GAPDH are indicated. Data represent one of two independent experiments with similar results.

(AgCAT-5) (Fig. 1*B*, *upper panel*), indicating that the 4.5-kb first intron fragment plays a major role in mediating the transcriptional activation effect of SOX9 on the aggrecan gene in TC6 cells. SOX9 reduced CAT activities by ~30% when both the promoter and intron were deleted (AgCAT-6) (Fig. 1*B*, *upper panel*).

As SOX9 is expressed specifically in cartilage but not in bone, it may require certain transcription factors or cofactors pertinent to a specific cellular background to enhance the 8-kb aggrecan gene fragment. To test this point, the AgCAT-8, AgCAT-5, and AgCAT-6 constructs were cotransfected with the SOX9 expression vector into osteoblast-like ROS17/2.8 cells. SOX9 overexpression decreased the transcriptional activity of the AgCAT-8 construct by ~80% in ROS17/2.8 cells. SOX9 suppression of the transcriptional activity of AgCAT-8 was lost when the 4.5-kb intron region was deleted (AgCAT-6 and AgCAT-5) (Fig. 1*B*, *middle panel*). Thus, SOX9 enhancement of aggrecan gene promoter activity was observed in TC6 cells, but not in ROS17/2.8 cells.

To compare SOX9 effects on AgCAT-8 activity in primary cultures of chondrocytes *versus* those in TC6 cells, we carried out experiments using primary chondrocytes prepared from ribs of newborn mice (16), primary osteoblasts prepared from calvariae of newborn mouse (17), and primary fibroblasts from adult mice. In contrast to the SOX9 enhancement of AgCAT-8 activities in TC6 cells, SOX9 suppressed AgCAT-8 activity in primary chondrocytes slightly (by 20%), although the effects were statistically significant ($p < 0.05$) and reproducible in two independent experiments (Fig. 1*B*, *lower panel*). In primary cultures of osteoblasts and fibroblasts, SOX9 affected AgCAT-8 activity slightly again (with 10–20% suppression) in a similar direction as seen in primary chondrocytes, although these SOX9 effects in primary osteoblasts and fibroblasts were not statistically significant in both of the two independent experiments (Fig. 1*B*, *lower panel*).

SOX9 Activates Transcriptional Activity via the Sry/Sox Consensus Sequence in TC6 Cells—SOX9 has been shown to enhance transcriptional activity via the *Sry/Sox* consensus sequence (6, 7, 27). However, it was not known whether the SOX9 activation of transcription via such consensus sequences could also be affected by the cellular background. To address this issue, the LUC reporter construct that carries four tandem copies of a 30-mer containing the *Sry/Sox* consensus sequence (AACAAAG) was cotransfected with the SOX9 expression vector into TC6 cells (24). SOX9 overexpression enhanced the transcriptional activity of the LUC reporter construct containing the *Sry/Sox* consensus sequence >10-fold in TC6 cells (Fig. 2). To examine SOX9 enhancement of transcriptional activity via the *Sry/Sox* consensus sequence in another cellular background, the LUC reporter construct containing the *Sry/Sox* consensus sequence was cotransfected with the SOX9 expression vector into ROS17/2.8 cells. The transcriptional activity of the *Sry/Sox* consensus sequence was not enhanced by SOX9 in ROS17/2.8 cells (Fig. 2).

Sox9 mRNA Expression and Its Up-regulation by RA in TC6 Cells—We next examined the endogenous levels of SOX9 expression in TC6 cells. As shown in Fig. 3, *Sox9* mRNA was expressed, although at relatively low levels, in TC6 cells. To investigate the regulation of *Sox9* mRNA expression, we examined the effect of calciotropic hormones and cytokines. The *Sox9* mRNA level was not affected by 48-h treatment with 4 ng/ml transforming growth factor β1, 10 ng/ml insulin-like growth factor, 10 ng/ml interleukin-1β, 100 nM vitamin D, or 100 nM parathyroid hormone. However, 1 μM RA specifically up-regulated *Sox9* mRNA expression in TC6 cells (Fig. 3). GAPDH mRNA levels (serving as a control) were not altered by RA, suggesting the specificity of the RA effects on *Sox9* gene expression in TC6 cells.

The dose dependence of the RA effect on *Sox9* mRNA expression in TC6 cells was examined at 0.01, 0.1, and 1 μM. The effect of 48 h of treatment with 1 μM RA on *Sox9* mRNA expression was investigated (Fig. 4). Again, RA at 1 μM did not affect GAPDH expression, indicating the specificity of the RA effect on *Sox9* gene expression.

Temperature Shift Effect on Sox9 mRNA Expression in Response to RA in TC6 Cells—TC6 cells are derived from articular cartilage of transgenic mice harboring the temperature-sensitive mutant tsA58 SV40 large T-antigen gene (14). Therefore, we examined the effect of temperature shift on *Sox9* mRNA expression in response to RA in TC6 cells. As shown in Fig. 5, the basal levels of *Sox9* mRNA expression were similar regardless of temperature (33 °C (permissive) and 39 °C (nonpermissive)) in TC6 cells. Furthermore, treatment with RA enhanced *Sox9* mRNA expression at 39 °C similarly to treatment at 33 °C (Fig. 5). These results indicate that the basal levels of SOX9 expression and the RA effects on its expression are similar between 33 and 39 °C.

RA Suppresses the Transcriptional Activity of the Aggrecan Gene Promoter/Enhancer in TC6 Cells—As SOX9 activates the 8-kb aggrecan gene fragment and RA enhances *Sox9* gene expression in TC6 cells, we wished to test whether RA enhances the transcriptional activity of the 8-kb aggrecan gene fragment. Contrary to our prediction, RA suppressed the transcriptional activity of AgCAT-8 by ~70%, although this was statistically not significant ($p = 0.055$). This suggests the parallel presence of RA-dependent suppressive pathway(s) that could overcome the RA-activated SOX9 activity on AgCAT-8 (Fig. 6).

RA Transactivates Gene Expression via the Sry/Sox Consensus Sequence in TC6 Cells—As the entire 8-kb aggrecan gene fragment could be regulated by many pathways or transcription factors other than SOX9, we tested whether at least the *Sry/Sox* consensus sequence could be activated by RA in TC6 cells. To examine whether RA enhances the transcriptional activity if only *Sry/Sox* consensus sequences are linked to the reporter gene in TC6 cells, we used the LUC reporter construct that carries four tandem copies of the 30-mer containing the AACAAAG consensus motif as described above. Treatment

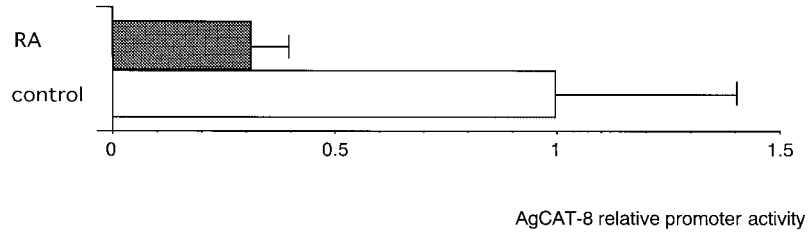

FIG. 6. **RA suppresses the transcriptional activity of the 8-kb aggrecan gene promoter/enhancer in TC6 cells.** The transcriptional activities of the AgCAT-8 reporter construct containing 8-kb promoter/first exon/first intron fragments of the aggrecan gene in the presence or absence of RA in TC6 cells were examined. TC6 cells were transfected with the AgCAT-8 construct and cultured in the absence or presence of 1 μM RA for 72 h. CAT activity was determined as described under "Materials and Methods." The experiment was conducted in three independently transfected cultures/group. Data are presented as means ± S.D. (n = 3).

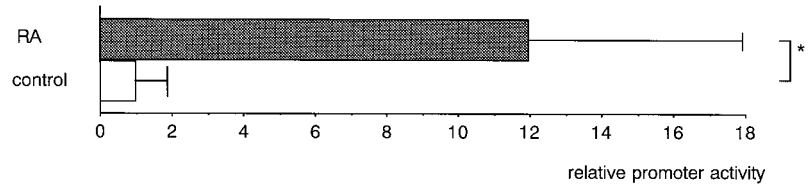

FIG. 7. **RA activates transcription via the *Sry/Sox* consensus sequence in TC6 cells.** The transcriptional activity of the AACAAAG concatamer-LUC reporter gene construct by RA in TC6 cells was examined. TC6 cells were cotransfected with 1 μg of reporter plasmid and cultured in the absence or presence of 1 μM RA for 72 h. Experiments were repeated twice using six independently transfected cultures. Representative data are shown. Data are presented as means ± S.D. (n = 6). *, $p < 0.01$ by unpaired Student's t test.

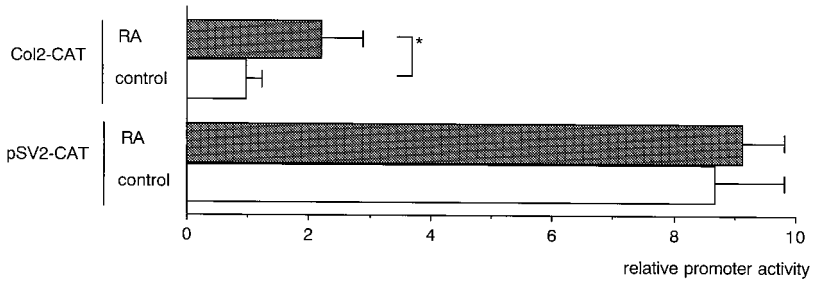

FIG. 8. **RA enhances the transcriptional activity of a reporter construct containing the type II procollagen gene enhancer in TC6 cells.** The RA effect on the transcriptional activity of the pCII4-C-CAT (Col2-CAT) reporter construct containing the chondrocyte-specific *Sry/Sox* consensus sequence within the enhancer region of the type II procollagen gene was examined. TC6 cells were transfected with 1 μg of reporter plasmid and cultured in the absence or presence of 1 μM RA for 72 h. The pSV2-CAT plasmid was used as a positive control. The transfected cells were incubated for 72 h. CAT activity was determined as described under "Materials and Methods." Experiments were repeated twice in triplicate with independent preparations of cell extracts. Representative data are shown. Data are presented as means ± S.D. (n = 3). *, $p < 0.01$ by unpaired Student's t test.

with RA at 1 μM enhanced by 12-fold the transcriptional activity of the LUC reporter construct containing the *Sry/Sox* consensus sequence (Fig. 7).

RA Enhances the Transcriptional Activity of the Type II Procollagen Gene in TC6 Cells—As we observed that SOX9 activated the transcriptional activity of the aggrecan gene promoter/enhancer in TC6 cells, we wished to test whether any other gene fragment that has been well characterized can respond to SOX9. Among the genes encoding cartilage-specific molecules, *SOX9* is known to enhance type II procollagen enhancer activity in chondrocytes (6, 7, 27). Therefore, we examined whether RA enhances type II procollagen enhancer activity. In TC6 cells, treatment with RA at 1 μM enhanced the activity of the pCII4-C-CAT (Col2-CAT) reporter construct containing a 1.6-kb intron fragment of the murine type II procollagen gene where the chondrocyte-specific *Sry/Sox* consensus sequence has been located (28) (Fig. 8). RA did not alter the activity of pSV2-CAT, indicating the specificity of its effect on the type II procollagen enhancer.

RA Suppresses Proliferation of TC6 Cells—As agents acting on the expression of the differentiation-related phenotype could reciprocally regulate proliferation, we examined the effect of RA on the proliferation of TC6 cells. The cells were cultured in the absence or presence of 1 μM RA for 1, 2, and 3 days. RA reduced TC6 cell numbers by 22% on day 2 and by 40% on day 3 (Fig. 9*A*). RA-mediated reduction of TC6 cell proliferation was observed in a dose-dependent manner after 3 days of treatment (Fig. 9*B*).

SOX9 Does Not Affect Alkaline Phosphatase Expression in ROS17/2.8 Cells and Primary Osteoblasts—To further examine the specificity of SOX9 action in chondrocytes, SOX9 was overexpressed in ROS17/2.8 cells and primary osteoblasts to see its effect on alkaline phosphatase mRNA expression. Endogenous levels of SOX9 were not detectable in these cells. SOX9 overexpression was confirmed by the presence of the *Sox9* mRNA band (Fig. 10), but it did not affect alkaline phosphatase mRNA expression in both ROS17/2.8 cells and primary osteoblasts. GAPDH mRNA levels served as a control and were not affected by SOX9 overexpression.

DISCUSSION

We demonstrate here that SOX9 enhances the transcriptional activity of the 8-kb fragment of the aggrecan gene. Bi *et al.* (11) reported that SOX9$^{-/-}$ cells do not express aggrecan in mouse chimeras, suggesting that SOX9 activates aggrecan gene expression *in vivo* as well. SOX9 has been shown to bind to chondrocyte-specific enhancer sequences in the first intron of the murine type II procollagen gene (6, 27) as well as the murine type XI procollagen gene (29) to activate transcription of these genes through these enhancers. Our observation pre-

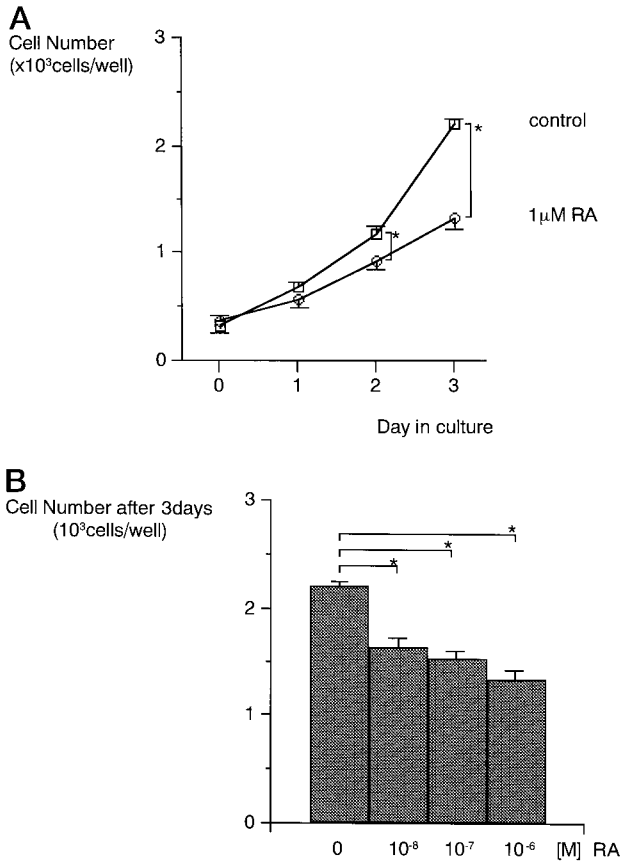

FIG. 9. **RA suppresses the proliferation of TC6 cells.** TC6 cells were seeded into four-well plates (2 cm^2/well) and cultured in α-MEM supplemented with 0.5% FBS in the absence or presence of RA. At the indicated time points, the cells were trypsinized and counted five times using Coulter counter. *A*, time course of the RA effects on cell number. RA at 1 μM significantly reduced the number of TC6 cell after 2 and 3 days of treatment. *B*, dose dependence of the RA effect on cell proliferation. RA reduced TC6 cell numbers in a dose-dependent manner after 3 days of treatment. Experiments were repeated twice using four independently treated cultures. Representative data are shown. Data are presented as means ± S.D. ($n = 4$). *, $p < 0.01$ by unpaired Student's *t* test.

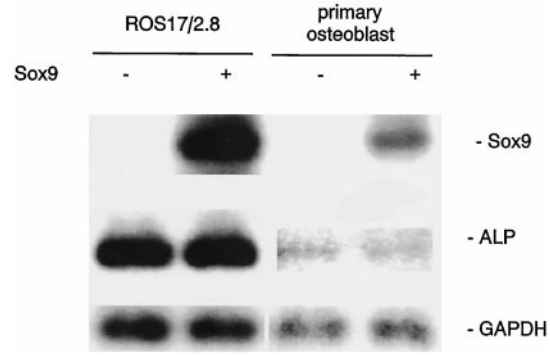

FIG. 10. **SOX9 effect on alkaline phosphatase mRNA expression in ROS17/2.8 cells and primary osteoblasts.** ROS17/2.8 cells and primary osteoblasts were seeded into 10-cm plates, and confluent ROS17/2.8 cells and primary osteoblasts were transfected with 10 μg of empty pSG5 vector (−) or 10 μg of SOX9 expression vector (+) using 20 μg of LipofectAMINE. After transfected cells were incubated for 48 h, total RNA was isolated and subjected to Northern blot analysis as described under "Materials and Methods." *Sox9*, alkaline phosphatase (*ALP*), and GAPDH mRNAs are indicated.

dicts that binding site(s) of SOX9 would exist within the 4.5-kb first intron fragment of the aggrecan gene. As support for this prediction, we found that SOX9 transactivates gene expression via the *Sry*/*Sox* consensus sequence in TC6 cells using a LUC reporter construct that carries four tandem copies of a 30-mer containing AACAAAG sequence derived from the enhancer region of a gene encoding CD3-ϵ, one of the T-cell antigen receptor family (24).

In this study, SOX9 enhancement of aggrecan gene promoter activity and SOX9 transactivation of gene expression via the *Sry*/*Sox* consensus sequence were observed in the cartilage-derived cell line TC6, but not in osteoblastic ROS17/2.8 cells. *In situ* hybridization studies indicate that *Sox9* is expressed in cells that express cartilage-specific phenotype-related genes such as aggrecan and the type II procollagen gene, but not in bone and other connective tissues (7). This association of *Sox9* expression and the aggrecan in *in situ* hybridization as well as our present study suggest that SOX9 is active in a specific cellular background. It is intriguing that SOX9 did not enhance, but suppressed gene transcription of the same reporter gene in osteoblastic ROS17/2.8 cells. It has been known that regulation of gene transcription is dependent on either tissue-specific positive or negative cofactors that modulate the functions of basic transcriptional machinery to render a variety of transcriptional regulation. It is intriguing to suspect that SOX9 will be functional in association with particular set(s) of transcriptional modulators in specific types of cells in different tissues. In fact, cells that do not express the type II procollagen gene such as those in genital ridges and a specific area in the embryonic heart also express *Sox9* (7). *Sox9* is also highly expressed in testicular Sertoli cells and is involved in differentiation of male gonads (30, 31). It is likely that SOX9 contributes to the function of these cells by controlling expression of diverse target genes. Kamachi *et al.* (32) proposed that the HMG domain of *Sox* genes is not sufficient to form a stable protein-DNA complex with SOX-binding DNA sequences by itself and that SOX proteins require binding partner factors unique to each SOX or SOX subfamily to achieve stable complex formation with the target DNA, which leads to transcriptional activation. Lefebvre *et al.* (33) suggested that in addition to SOX9, L-SOX5 and SOX6 cooperate with each other in expression of chondrocyte-specific genes.

The diversity in the SOX9-specific regulation may also be dependent on the small difference in the sequences flanking the *Sry*/*Sox* consensus motif. Bell *et al.* (6) reported that SOX9 fusion protein bound to the AACAAAG sequence derived from the enhancer region of a CD3-ϵ gene and that this complex was supershifted by anti-SOX9 antibody. Mertin *et al.* (34) reported that binding activity between SOX9 fusion protein and the AGAACAAAGC sequence derived from the enhancer region of CD3-ϵ was half of the binding activity between SOX9 fusion protein and the preferred DNA-binding site of SOX9 (AGAACAATGG), which was identified using a random oligonucleotide selection assay. Overall, a difference in SOX9 functions in the background of TC6 *versus* ROS17/2.8 cells may be due to such different sets of the partner factors, including L-SOX5, SOX6, and/or as yet unknown cofactors.

Against our prediction, SOX9 slightly suppressed AgCAT-8 activity in primary chondrocytes (by 20%). These effects were small although statistically significant ($p < 0.05$) and reproducible in two independent experiments (Fig. 1*B*, *lower panel*). We assume that this difference in the SOX9 regulation of AgCAT-8 in TC6 cells *versus* primary chondrocytes could be related to the difference in the endogenous levels of SOX9 expression. Northern blot analysis indicated that primary chondrocytes express *Sox9* mRNA at severalfold higher levels than TC6 cells (data not shown). SOX9 overexpression enhanced AgCAT-8 activity in TC6 cells, possibly due to the low SOX9 background in these cells, whereas SOX9 overexpression

in the presence of high levels of SOX9 may be suppressive, as competition against cofactors may exist.

Although SOX9 decreased AgCAT-8 transcriptional activity by ~80% in ROS17/2.8 cells, SOX9 virtually did not affect AgCAT-8 activity in primary osteoblasts. We could not detect *Sox9* mRNA expression by Northern blot analysis in either ROS17/2.8 cells or primary osteoblasts. Therefore, diverse cellular backgrounds (primary osteoblasts *versus* ROS17/12.8 cells) may affect AgCAT-8 activity differently in response to SOX9 overexpression. Overall, certain differences exist between TC6 cells and primary chondrocytes with regard to the SOX9 regulation of aggrecan gene expression, possibly due to the diversities in cellular backgrounds that would include those in the sets of transcription factors and cofactors.

RA plays an important role not only in pattern formation during embryogenesis (35–37), but also in regulation of chondrocytes in cultures. RA treatment decreases expression of the type II procollagen gene in primary chondrocytes (28, 38) and suppresses the enhancer activity of pCII4-C reporter plasmid containing the promoter and enhancer regions of the type II procollagen gene in primary chondrocytes prepared from sterna of day 15 chickens (20). In contrast, RA stimulated the enhancer activity of the pCII4-C reporter plasmid in TC6 cells, where RA enhanced mRNA levels of *Sox9*, which regulates the type II procollagen gene directly (6, 27). This apparent diversity in the responses to RA could be due to several possibilities. For instance, responsiveness to RA may depend on chondrocyte subtypes; however, primary chondrocytes consist of heterogeneous subpopulations. On the other hand, TC6 cells consist of homogeneous population and represent one of the subpopulations in chondrocytes at a certain stage, but not all. RA may up-regulate chondrocytic phenotype-related genes such as *Sox9* and type II procollagen in this subtype specifically. As another possibility, the apparent dissociation of RA effects on the 1.6-kb fragment of the type II collagen gene *versus* those on the transcriptional activity of the 8-kb fragment of the aggrecan gene in TC6 cells could be due to the diversities in the interaction of different sets of transcription factors that are binding to their cognate DNA elements present in various combinations within these two promoters.

Temperature shift experiments indicate that basal levels of *Sox9* expression and RA effects on its expression are similar between 33 and 39 °C, whereas at 39 °C, SOX9 did not affect AgCAT-8 activity in TC6 cells, in contrast to its enhancement at 33 °C (data not shown). These observations reveal that both a similarity and a difference exist between 33 and 39 °C cultures of TC6 cells. Chondrocytes at different maturation stages share certain common phenotypes, whereas chondrocytes at each of the specific stages such as proliferating chondrocytes or hypertrophic chondrocytes exhibit specific features. Our observations on the similarity and the difference between the TC6 cells cultured at 33 and 39 °C could represent these common and specific features, respectively.

Altogether, we demonstrate in this study that SOX9 activates aggrecan promoter/enhancer transcription in TC6 cells. Our results also suggest that RA enhances *Sox9* expression and, in turn, SOX9 transactivates the type II procollagen gene via the *Sry*/*Sox* consensus sequence in TC6 cells.

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SOX9 Enhances Aggrecan Gene Promoter/Enhancer Activity and Is Up-regulated by Retinoic Acid in a Cartilage-derived Cell Line, TC6

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